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Applicant: Lu WANG and Robert LUHM

Title: PRIMERS, METHODS and KITS FOR AMPLIFYING OR DETECTING
HUMAN LEUKOCYTE ANTIGEN ALLELES

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☐ Applicant claims small entity status under 37 CFR 1.27(c)(1).

Enclosed are:

- ☒ Specification, Claims, and Abstract (64 Pages Total: Specification - 58 pages; Claims - 3 pages; Abstract - 1 page; Drawings - 2 pages).
- ☒ Informal drawings (2 sheets, Figs. 1A and 1B, and 2A, 2B, 2C and 2D).
- ☐ Small Entity statement(s).
- ☒ Application Data Sheet (37 CFR 1.76).

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HUMAN LEUKOCYTE ANTIGEN ALLELES

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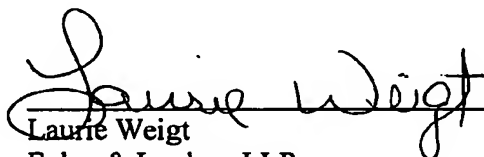
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**PRIMERS, METHODS AND KITS FOR AMPLIFYING OR
DETECTING HUMAN LEUKOCYTE ANTIGEN ALLELES**

FIELD OF THE INVENTION

This invention relates to the typing of human leukocyte antigen (HLA) alleles. More particularly, the present invention relates to the typing of HLA alleles using primer pairs, methods and kits that amplify all of the HLA alleles at a particular locus.

BACKGROUND

A major focus of tissue typing and disease association centers around the human leukocyte antigen (HLA) genes and the alleles encoded by these genes. The HLA alleles encompass the most diverse antigenic system in the human genome, encoding literally hundreds of alleles that fall into several distinct subgroups or subfamilies. Unfortunately, because the HLA alleles are commonly closely related, standard techniques for DNA typing have often proven inadequate in resolving many of these alleles.

The human leukocyte antigen complex (also known as the major histocompatibility complex) spans approximately 3.5 million base pairs on the short arm of chromosome 6. The HLA antigen complex is divisible into 3 separate regions which contain the class I, the class II and the class III HLA genes. The genes of class I and class II encode highly polymorphic cell-surface molecules that bind and present processed antigens in the form of peptides to T-lymphocytes, initiating both cellular and humoral immune responses. Thus, the class I and class II HLA genes play a major role in the recognition of self. The involvement of HLA peptides in this important immune function means that class I and class II HLA matches between individuals are generally sought prior to tissue transplantation in order to reduce rejection by either the host or the transplanted tissue.

In humans, the class I HLA genes encompass about 2000 kb and code for about 20 distinct genes. Each of the HLA class I genes is composed of eight exons and seven introns and the sequences of these exons and introns are highly conserved. Allelic variations mostly occur in exons 2 and 3, which are flanked by noncoding
5 introns 1, 2, and 3. Exons 2 and 3 encode the functional domains of the molecules. Within the class I region exist genes encoding the well characterized class I MHC molecules designated HLA-A, HLA-B and HLA-C. In addition, there are nonclassical class I genes that include HLA-E, HLA-F, HLA-G, HLA-H, HLA-J and HLA-X. The class I molecules, HLA-A, -B, and -C, are found on most nucleated cells.

10 The class II molecules are encoded in the HLA-D region. The HLA-D region contains several class II genes and has three main subregions: HLA-DR, -DQ, and -DP. The class II cell-surface glycoproteins consist of HLA-encoded alpha, and beta-chains, associated as heterodimers on the cell surface of antigen-presenting cells such as B-cells and macrophages. Class II peptides serve as receptors for processed
15 peptides. Both the HLA-DQ and -DP regions contain one functional gene for each of their alpha and beta-chains. The HLA-DR subregion contains one functional gene for the alpha-chain; the number of functional genes for the beta-chain varies from one to two according to the haplotype.

The loci constituting the MHC are highly polymorphic, that is, many
20 forms of the gene or alleles exist at each locus. Several hundred different allelic variants of class I and class II MHC loci have been identified in humans.

The HLA class I and class II regions play a major role in determining whether transplanted tissue will be accepted as self (histocompatible) or rejected as foreign (histoincompatible). It has been demonstrated that matching of donor and
25 recipient HLA-DR and DQ alleles prior to allogeneic transplantation has an important influence on allograft survival. Therefore, HLA-DR and DQ matching is now generally undertaken as a clinical prerequisite for renal and bone marrow transplantation as well as cord blood applications. In many transplant situations, HLA class I loci and allelic matching is also sought. In order to fully match samples, up to
30 6 different class I loci and up to 12 different class II loci must be matched for individual alleles in a single transplant recipient.

Until recently, HLA matching has been confined to serological and cellular typing. For instance, in the microcytotoxicity test, white blood cells from the potential donor and recipient are distributed in a microtiter plate and monoclonal antibodies specific for class I and class II MHC alleles are added to different wells.

5 Thereafter, complement is added to the wells and cytotoxicity is assessed by uptake or exclusion of various dyes by the cells. If the white blood cells express the MHC allele for a particular monoclonal antibody, then the cells will be lysed upon addition of complement and these dead cells will take up the dye. A general review of this method can be found in Terasaki *et al.* *Nature*, 204: 998 (1964). Unfortunately,
10 serological typing is frequently problematic, both because live cells are required and because of the lack of availability of alloantisera and its general crossreactivity. A high degree of error and variability is also inherent in serological typing, which ultimately affects transplant outcome and survival (Sasazuki *et al.*, *New England J. of Medicine* 339: 1177-1185 (1998)). Therefore, DNA typing is becoming more widely
15 used as an adjunct, or alternative, to serological tests.

Analysis of HLA specificities using DNA provided a new approach to defining the polymorphic differences in the HLA genes. Thus, rather than looking at differences in the expressed protein, polymorphism can be characterized at the nucleotide level using DNA typing. Initially, the most extensively employed DNA
20 typing method for the identification of the HLA loci and alleles was restriction fragment length polymorphism (RFLP) analysis. However, this method for HLA DNA typing suffers from a number of inherent drawbacks such as not being able to generally detect polymorphism within the exons that encode functionally significant HLA class II epitopes, and having to rely instead upon the strong (but not perfect) linkage between
25 allele-specific nucleotide sequences within these exons and restriction endonuclease recognition site distribution within surrounding, generally noncoding, DNA.

The polymerase chain reaction (PCR) dramatically changed the ability to identify specific HLA loci and alleles as the nucleotide level. In tissue typing, PCR is used to amplify the polymorphic regions of HLA genes. This HLA PCR amplicon
30 can then be analysed for its polymorphic differences in order to establish the tissue type. A number of analysis approaches have been developed, including heteroduplex analysis of PCR products (Clay *et al.*, 1994), single-stranded conformational

polymorphism analysis of the PCR product (PCR-SSCP; Yoshida *et al.*, 1992), the use of sequence specific primers in PCR reaction (PCR-SSP; Olerup *et al.*, 1991), the use of PCR in combination with sequence-specific oligonucleotide probing (PCR-SSOP; Saiki *et al.*, 1986) and probing by reverse dot-blot (Saiki *et al.*, 1989). These approaches, used singly or in combination, have all been applied as DNA-based methods for tissue-typing of class I and class II HLA specificities.

A popular approach to tissue-typing of HLA alleles has been the hybridization of PCR amplified products with sequence-specific oligonucleotide probes (PCR-SSO) to distinguish between HLA alleles (see Tiercy *et al.*, Blood Review 4: 9-15 (1990)). This method requires a PCR amplicon of the HLA locus of interest be produced and then dotted onto nitrocellulose membranes or strips. Then each membrane is hybridized with a sequence specific probe, washed, and analyzed by exposure to x-ray film or by colorimetric assay. Probes are made to the allelic polymorphic area responsible for the different HLA alleles. To obtain accurate results, each sample must be hybridized and probed at least 100-200 different times for complete Class I and II typing. Thus, a common drawback to this methods is its relatively high complexity and resulting high cost. In addition, the necessity for sample transfers and washing steps increases the chances that small amounts of amplified DNA might be carried over between samples, creating the risk of false positives.

Recently, researchers have begun using sequence based typing (SBT) to identify the loci and alleles of HLA genes. A background on the use of SBT in tissue typing can be found in U.S. Patent Appl. No.: 20030017458 and Santamaria *et al.*, 1992 and 1993. In SBT methods, the HLA locus or allele is amplified using a technique such as PCR and then the resulting amplicon is sequenced to unambiguously identify the HLA locus and allele. The method does not require that the amplicon be transferred to a membrane for hybridization. Thus, SBT methods dramatically reduce both the cost and the complexity seen with SSO methods. Unfortunately, the SBT methods currently available in the art do not allow different HLA loci to be identified using similar reaction conditions. Typically, class I and class II HLA have been segregated from each other during SBT because amplification conditions have been quite different. Not having a uniform protocol for both class I and class II increases both the time and expense required for HLA typing using SBT.

Further, most amplification methods, include SBT methods disclosed in the art use the amplification of housekeeping genes as amplification reaction controls. Unfortunately, problems exist in using the amplification of housekeeping genes as controls because amplification of a housekeeping gene fails to make it possible to recognize whether the lack of HLA amplification means the sample does not have that particular locus or allele or simply that the amplification of the HLA locus or allele failed to proceed.

In view of the foregoing, what is needed in the art is a convenient method of determining genomic information from a highly polymorphic system such as the HLA class I and class II regions. Specifically, a need exists to be able to identify both class I and class II HLA loci using similar reaction conditions. A further need exists to be able to use the target HLA loci or allele as an amplification reaction control in order to be able to accurately determine whether a particular HLA loci or allele exists in a sample.

SUMMARY OF THE INVENTION

In one embodiment a primer set for identifying a human leukocyte antigen allele at a specific locus is described. In some embodiments, the primer set consists of primers capable of amplifying all HLA alleles of an HLA locus. The primer set may also comprise a control primer pair that will produce an HLA control amplicon of predetermined size from an HLA allele if the HLA allele is present in the sample. In certain embodiments, the primer set is capable of simultaneously amplifying a plurality of one or more portions of a class I HLA allele. In some cases, these plurality of HLA alleles will belong to the same HLA locus.

These primer sets can also encompass primers capable of sequencing an HLA allele. In some cases, these sequencing primers comprise 5' regions that are not complementary to the HLA allele.

Based on these primers and primer sets, methods of detecting HLA alleles using the primers and primer sets are described. Kits for carrying out these methods are also provided in some embodiments. These kits can include instructions

for carrying out the methods, one or more reagents useful in carrying out these methods, and one or more primer sets capable of amplifying all HLA alleles.

Objects and advantages of the present invention will become more readily apparent from the following detailed description.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B demonstrate agarose gels illustrating amplification results obtained by using the primers and primer set of the present invention. FIGS. 1A and 1B exhibit positive amplification of HLA A locus alleles and HLA B locus alleles, respectively.

10

Figures 2A-2D show sequencing electropherograms from the alleles amplified and sequenced in the examples.

DETAILED DESCRIPTION

The present invention provides a highly accurate and efficient HLA class I and class II sequence-based typing method that is rapid and reliable. The primers, primer sets, and methods of the present invention not only strengthen amplification and sequencing reaction robustness, but they also provide specificity and product stability not seen with other primers or methods of HLA sequence-based typing. Moreover, the primers, primer sets and methods of the present invention allow similar fractionation times for sequencing of the amplification product regardless of the sequence or size of the initial DNA template. Generally, the primers, primer sets and methods of the present invention allow all HLA loci to be amplified and sequenced under identical or substantially similar reaction conditions.

Described herein are primers, primer sets, methods, and kits for amplifying and/or detecting human leukocyte antigen alleles. Some embodiments of the present invention provide primer sets and methods for amplifying HLA alleles from a particular HLA locus. Other embodiments include primer sets capable of amplifying a portion of HLA Class I alleles using the same reaction conditions. Generally, the primer sets demonstrated below can be used to amplify any HLA alleles that are present in a sample. Accordingly, the present primers, primer sets,

methods and kits can be used for research and clinical applications for any HLA associated disease, disorder, condition or phenomenon.

5 In one aspect, the present invention comprises a primer set with primers that are capable of amplifying all human leukocyte antigen (HLA) alleles of a locus, as well as a control primer pair that produces an HLA control amplicon. As used herein, a HLA control amplicon generally refers to the product produced from the amplification of the HLA allele using the control primers. As set forth in more detail below, it is advantageous to use the individual HLA allele as a control. Generally, the molecular weight of the control amplicon will be predetermined, 10 meaning that the expected size of the product from the control reaction will be known prior to the reaction. The size of the control amplicon is not particularly limiting and can be any size capable of amplification and detection, including but not limited to less than 500, 500-600, 600-700, 700-800, 800-900, 900-1000, or more than 1000 base pairs in length. In one embodiment, the HLA control amplicon can quickly be 15 checked using gel electrophoresis based on molecular weight, which allows quick determination of the success of the amplification reaction. When multiple HLA loci are being amplified with the primer sets of the present invention, it is understood that a control primer pair will be included for each loci but will be common to all or substantially all of the HLA alleles at a particular loci.

20 Still another aspect of the invention provides for a primer set that can simultaneously amplify a plurality of a portion of class I alleles using the same or substantially similar reaction conditions. In this embodiment, reaction conditions generally encompass the conditions, such as PCR conditions, used to amplify the particular HLA class I alleles.

25 Surprisingly and unexpectedly, it was discovered that the primer sets of the invention allow for identification of all HLA alleles of a particular HLA locus using sequence-based typing. Thus, another aspect of the invention encompasses a method of amplifying a HLA allele using the primer sets above and then sequencing the amplicon using a separate primer set of sequencing primers.

30 Amplicons amplified using the primers or primer sets of the invention can be sequenced using specialized sequencing primers. In some embodiments, the sequencing primers will contain 5' portions that are not complementary to the HLA

allele of interest. The advantages concerning the use of sequencing primers containing non-homologous bases on the 5' portion are set forth in further detail below. The sequencing primers can be used in methods of sequence based typing. For example, an individual sequencing primer can be used in performing a sequencing
5 reaction and subsequently determining the sequence of one or more HLA alleles. It was unexpected discovered that the sequencing primers of the present invention provide for improved resolution of many HLA alleles. In some embodiments, complete exon sequence can be determined using the amplification and sequencing primers of the present invention.

10 An example of protocol that demonstrates embodiments of certain methods of the present invention generally includes one or more of the following steps: amplification, detection of the amplification products, treatment of the amplicons to remove unused primers, sequencing, treatment of the sequencing product to remove excess terminators, resuspension and denaturation of the cleaned
15 sequencing product, running the sequencing product on a sequencer, analysis of the results of the sequencing program, and allele assignment.

Although the general concepts, such as amplifying and sequencing the HLA allele using primers specifically targeting the exons that used with the primers, primer sets, methods and kits of the present invention are applicable to any type of
20 closely related alleles, in one embodiment methods focus on identifying HLA alleles. The alleles of the HLA loci are classified as Class I, generally encompassing HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G alleles, or Class II, generally encompassing HLA-DRA, HLA-DRB1, HLA-DRB2-9, HLA-DQA1, HLA-DQB1, HLA-DPA1, HLA-DPB1, HLA-DMA, HLA-DMB, HLA-DOA and HLA-DOB
25 alleles. Over a hundred identified alleles fall these loci and many of these alleles are closely related and can differ in sequence by only one, or a few, nucleotide positions. HLA loci and alleles are well known in the art. For a review of the HLA genes, see Schreuder *et al.*, Tissue Antigens, 58:109 (2001) and the references disclosed therein.

One primer set includes primers that are capable of amplifying all
30 HLA alleles of an HLA allele subset or locus and also a control primer pair that is capable of producing an HLA amplicon of a defined size if the one or more HLA alleles are present in the sample. This primer set utilizes the target HLA allele as the

template for the control primer pair and provides an indication of the presence of absence of one or more HLA alleles. Any HLA alleles of the HLA allele subset or locus are also the template for the other primers in the primer set. In some embodiments, the control primer will not use an HLA gene as a template but will
5 amplify a ubiquitous gene in the sample. One of skill in the art will understand that in these embodiments, control primers to ubiquitous genes may be used with the primers and primer sets that amplify HLA alleles. Generally, primers to any gene that can serve as an adequate reaction control may be used. A non-limiting example includes primers that amplify the GAPDH housekeeping gene.

10 In yet further embodiments, the control primers can be used not only as controls but also to resolve alleles of HLA loci. For example, by designing primers to exon 3 of the DQB1 HLA loci, the 0201 and 0202 alleles of the locus can be resolved. The skilled artisan will understand that control primers that amplify regions of a HLA allele can be used to resolve alleles in loci other than DQ. By adding sequencing tails,
15 or portions of nucleotide sequence that can be recognized by sequencing primers, the control amplicon can be sequenced allowing further allele resolution.

As will be understood by the skilled artisan, in order to provide an effective control, the portion of the HLA allele amplified by the control primer pair is typically common to all or substantially similar to all HLA alleles being tested. In
20 some embodiments, particularly when the target HLA locus is HLA A or HLA B, the portion of the HLA allele amplified by the control primer pair comprises all of exon 4 and beyond exon 4. In other embodiments, the control primer pair amplifies all of exon 4 and all of exon 5 of the HLA allele. Thus, in these embodiments, the primer set can be used in an amplification reaction to amplify an HLA allele and also provide
25 a control. In certain embodiments, the control primer pair not only provides an indication of the efficiency of any HLA allele amplification that occurs in the amplification reaction, but also prevents false positive results. Using control primers that amplify an HLA allele are advantageous as they provide for a mechanism to ensure that DNA has in fact been added to the amplification reaction. This type of
30 control allows for an easy determination of whether the reaction is truly negative or is only negative because of a technical error. For example, if the results of the amplification provide an amplicon but lack the control amplicon, then the amplicon is

likely a false positive. In contrast, if the control amplicon is also present, then the amplification produced a positive result.

In some embodiments, the primers of the control primer pair are selected such that any amplicon they produce will have a substantially constant size, such as less than 500, 500-600, 600-700, 700-800, 800-900, 900-1000 or greater than 1000 base pairs, regardless of the HLA alleles that are present in the sample. As long as the control primer pair does not interfere with the control parameters, the control primer pair can span a region with or without polymorphic positions. Accordingly, the portion of the HLA allele amplified by the control primer pair can have base polymorphisms as well as insertions or deletions.

Another primer set of the present invention contains a plurality of primer pairs, typically in the same solution, that are capable of simultaneously amplifying a plurality of class I HLA alleles. As such, this primer set is capable of performing a successful multiplex amplification for all HLA alleles of an HLA allele subset or locus, such as the A locus, B locus or DR loci. In some embodiments, this multiplex amplification is achieved while still maintaining individual locus specificity because the product sizes produced from the amplification of individual loci differ in size. Because each HLA locus is physically distinct, some being separated by large distances, in some embodiments all loci could be amplified in a single multiplex reaction which amplifies all or a selected subgroup of clinically significant loci. In embodiments that amplify all loci in a single multiplex reaction, locus specific sequencing primers would preferentially be employed.

In some embodiments, a single multiplex amplification reaction can be run for all major HLA loci, e.g. HLA A, HLA B, HLA C, etc. in a single vessel. In other embodiments, separate amplification reactions will be run for each HLA loci. Any of these separate amplification reactions can employ a non-multiplex approach that produces amplicons encompassing all of the desired locus or a multiplex approach to produce amplicons that encompass shorter segments of the amplified locus. Thus, in these embodiments, the products of the reaction can take two forms. The first form is a single large amplicon that encompasses as much of the locus as desired. The second form encompasses amplicons covering the same sequence of the HLA locus but instead of a single large amplicon, multiple amplicons that

individually cover smaller portions of the HLA loci sequence result. In some embodiments, the multiplex reaction results in a stronger amplification signal. Both types of reactions can be run simultaneously or subsequently as desired.

Primer sets having combinations of the above characteristics can also
5 be used. As a non-limiting example, individual primer pairs in the primer sets can be used with controls to specifically target individual amplicons or individual HLA loci.

As stated above, in certain embodiments, the primers and primer sets of the invention can be used in reactions that amplify HLA alleles. Suitable amplification reactions include those that proceed both linearly and exponentially.
10 The present methods are also simplified as they can provide a common sequencing protocol for all HLA loci. The skilled artisan will easily understand the advantages, such as decreased sequencing primer costs, in having uniform sequencing protocols for all HLA loci. The above primer sets will contain primers that are sufficient to amplify all alleles of an HLA locus, such as HLA A, HLA B, etc. in a single reaction.
15 Accordingly, the primers can be targeted to hybridize to non-specific regions of the chosen HLA locus so that all different HLA alleles can be successfully amplified. The primers can also be located so that the HLA amplicon resulting from the primers spans enough polymorphic positions of the locus so that individual alleles can be identified in a subsequent sequencing or typing reaction utilizing the HLA amplicon.
20 The present primer pairs can also be used individually to identify a single HLA allele, as desired.

In certain embodiments, where HLA A locus alleles are being amplified, the present primers can be selected to provide a single amplicon that includes exons 2, 3 and 4. Where HLA B locus alleles are being amplified, different
25 primer sets can be used to produce dual amplicons that cover exons 2, 3 and 4. Some embodiments of the present primers, primer sets, methods and kits utilize two separate amplifications in the B locus to reduce the number of potential heterozygotic combinations. This results in simplified sequence analysis, and the position of split primers on the B locus results in resolution of more of the locus, thus further reducing
30 the number of resultant ambiguities. For example, these advantages can be achieved by amplifying the regions from exon 1 to intron 3 and intron 3 to exon 5 as two separate products simultaneously in one amplification mix. This results in a much

more robust amplification than amplification of a single product. Amplifying the HLA B locus as two separate products is advantageous over a single product as a single product is frequently weak, making it difficult to discern using detection methods such as agarose electrophoresis. This difficulty is particularly prominent
5 when modified nucleotides are required.

In HLA DR locus (Class II) amplification, one embodiment provides for a primer set that allows for eleven group specific amplifications that achieve resolution of DRB1, DRB3, DRB4, and DRB5 within exon 2. Although the primer sets are envisioned to resolve regions outside of DR locus exon 2, resolving exon 2
10 currently has special significance as the standard convention in the transplant community is that only resolution of exon 2 is relevant for DR tissue matching. The skilled artisan will understand that this may, and will, likely change with time, as several ambiguities remain unresolved by only using an exon 2 resolution approach.

Some of the present embodiments also provide complete locus
15 resolution by employing locus specific primers located in the 5' and 3' untranslated areas of the gene. Complete locus resolution can be approached in two distinct ways. The first can utilize an expansion and enhancement of the multiplex approach, where the entirety of the locus would be amplified from genomic DNA resulting in two or more products created simultaneously. This could be accomplished by two larger
20 products, or potentially three smaller products. Second, amplification of the locus using RNA as the starting template through simultaneous RT/PCR could provide total locus coverage with a manageable single product. This would provide ultimate resolution of HLA types.

General and non-limiting position strategies for the HLA allele
25 amplification primers are set forth in Table 1. In some embodiments, the primer hybridization positions shown in Table 1 can be varied by one, two, five, ten, twenty or more positions, or any number of positions between one and twenty, either upstream or downstream, and still provide acceptable results. As used herein, acceptable results generally encompass results where there will be resolution of the
30 functional aspect of the HLA locus with sequence of sufficient quality to provide unambiguous HLA typing for that locus. The skilled artisan will understand that unambiguous HLA typing as an acceptable result does not mean the complete

elimination of ambiguities, rather it means that the data generated is unambiguous. Typically, in embodiments where the primer hybridization position is moved upstream of the position illustrated in Table 1, additional bases that hybridize to the HLA allele further upstream of the primer demonstrated in Table 1 will be added. Similarly, when
5 the hybridization position is moved downstream, then bases are added to the primer that hybridize to the HLA allele downstream. In many embodiments, when the hybridization position of the primer demonstrated in Table 1 is moved either upstream or downstream, this will be accompanied by removal of bases from the end of the primer opposite the end moved either upstream or downstream.

10 In certain embodiments, the amplification primers will have a 5' portion containing non-homologous sequence or sequence that does not hybridize to the HLA allele, but can provide enhanced specificity of amplification of the target sequence. As a non-limiting theory, it is believed that this increased specificity results from being able to lower the strength of the binding to more than one HLA
15 locus by providing a primer with initial weaker binding. However, a robust product is still obtained because once the amplification reaction begins to proceed, the non-homologous sequences are incorporated into the product, thus providing homologous sequence when subsequent primers bind during further amplification.

In some embodiments, the 3' terminus of the primers of the primer sets
20 are capable of being extended by a nucleic acid polymerase under appropriate conditions and can be of any length, for example ranging from about 5 nucleotides to several hundred. Preferably, the primer oligonucleotides will have a length of greater than 10 nucleotides, and more preferably, a length of from about 12-50 nucleotides, such as 12-25 or 15-20. In some embodiments, the primer oligonucleotides can be
25 chosen to have a desired melting temperature, such as about 40 to about 80°C, about 50 to about 70°C, about 55 to about 65°C, or about 60°C. In any case, the length of the primer is sufficient to permit the primer oligonucleotides to be capable of hybridizing to the target molecule. The sequence of the primer oligonucleotide is selected such that it is complementary to a predetermined sequence of the target molecule.

30 The present primers and primer sets can be used in any method where nucleic acid primers find utility. For example, the primers are readily applicable to

RT PCR of HLA mRNA for expression analysis because they target exon regions.

The present primers can also be extended to, as yet, unknown HLA alleles. The skilled artisan will understand that the present primers and primer sets may be used to amplify RNA, DNA and/or cDNA. During amplification, the type of nucleic acid amplified by the primers and primers sets is not particularly limiting as long as the primers can hybridize and amplify the target nucleic acid in the sample. The skilled artisan will understand that sample from which the nucleic acid to be amplified derives can encompass blood, bone marrow, spot cards, RNA stabilization tubes, forensic samples, or any other biological sample in which HLA alleles can be amplified. Generally, the sample to be detected can be obtained from any suitable source or technique. The nucleic acid may also be isolated from the sample using any technique known in the art. In many embodiments, the nucleic acid will not be isolated from the sample before the amplification reaction. In other embodiments, the nucleic acid will be isolated from the sample prior to amplification.

One example of an assay where the present primer pairs find use includes a detection assay or method for identifying an HLA allele in a sample having, or suspected of having an HLA allele. In such an assay, generally, the sample will be contacted with the primers or primer set under conditions such that individual primer pairs, either alone or in a primer set, will amplify the HLA locus or allele for which the primer pair is specific, if that locus or allele is present in the sample. The presence or absence of the amplicon can then be determined or detected by standard techniques, such as separation techniques including electrophoresis, chromatography (including HPLC and denaturing-HPLC), or the like. Exemplary techniques for performing these assays are described in the examples section. As will be recognized by the skilled artisan, the production of an amplicon will indicate the presence of an HLA locus or allele in a sample. Accordingly, the presence or absence of an amplicon can be correlated with the presence or absence of the HLA locus or allele in the sample. In many embodiments, the presence or absence of an amplicon will be compared to the presence or absence of a control amplicon. In other embodiments, amplicons may be sent directly for sequencing without first determining or detecting if an amplicon is present in the sample by comparison to a control amplicon.

In many embodiments, the primers or primer sets will first be used in amplification reactions in order to identify HLA alleles. In many embodiments, nucleic acid amplification will take place using the polymerase chain reaction. Typically, nucleic acid amplification or extension involves mixing a target nucleic acid
5 with a "master mix" containing the reaction components for performing the amplification reaction and subjecting this reaction mixture to temperature conditions that allow for the amplification of the target nucleic acid. The reaction components in the master mix can include a buffer which regulates the pH of the reaction mixture, one or more of the four deoxynucleotides (dATP, dCTP, dGTP, dTTP - preferably present
10 in equal concentrations), that provide the energy and nucleosides necessary for the synthesis of DNA, primers or primer pairs that bind to the DNA template in order to facilitate the initiation of DNA synthesis and a DNA polymerase that adds the deoxynucleotides to the complementary DNA strand being synthesized. The polymerase used in the present methods and kits is not particularly limited, and any
15 suitable polymerase can be used. Examples of suitable polymerases include thermostable polymerase enzymes, such as the *Thermus aquaticus* (Taq) polymerase. Generally, preferred polymerases for use with the present primers, primer sets, methods and kits have low error rates.

In the polymerase chain reaction, a typical thermal cycling reaction
20 used in DNA amplification has a temperature profile of thermal cycling that involves an initial ramp up to a predetermined, target denaturation temperature that is high enough to separate the double-stranded target DNA into single strands. Generally, the target denaturation temperature of the thermal cycling reaction is approximately 91-97°C, such as 94°C-96°C, and the reaction is held at this temperature for a time period
25 ranging between 20 seconds to two minutes. Then, the temperature of the reaction mixture is lowered to a target annealing temperature which allows the primers to anneal or hybridize to the single strands of DNA. Annealing temperatures can vary greatly depending upon the primers and target DNA used. Generally, annealing temperatures range from 58°C-70°C depending on the sequence sought to be
30 amplified. Next, the temperature of the reaction mixture is raised to a target extension temperature to promote the synthesis of extension products. The extension

temperature is generally held for approximately two minutes and occurs at a temperature range between the annealing and denaturing temperatures. This completes one cycle of the thermal cycling reaction. The next cycle then starts by raising the temperature of the reaction mixture to the denaturation temperature.

- 5 Typically, the cycle is repeated 25 to 35 times to provide the desired quantity of DNA. As will be understood by the skilled artisan, the above description of the thermal cycling reaction is provided for illustration only, and accordingly, the temperatures, times and cycle numbers can vary depending upon the nature of the thermal cycling reaction and application. These variations can easily be determined
10 without undue experimentation by one skilled in the art.

- In certain embodiments, the present amplifications are preferably performed with a reaction volume and amount that is sufficient to perform a separation or detection step in addition to providing enough amplified product to perform a sequencing reaction. Typically, amplification reactions having 25 μ l or
15 more total volume are sufficient. The skilled artisan understands the advantages of having reaction volumes that are large enough to allow a separation or detection step before the sequencing step. For example, detection steps allow for the quick and easy determination of the success of an amplification reaction before going forward with the more expensive and time consuming sequencing reaction. However, some
20 embodiments may include only enough amplification reaction volume to subject the resulting amplicon to sequencing.

- After the HLA locus is amplified, the specific alleles of the locus can then be determined by any method or assay known in the art. One such method is a sequencing reaction, for example the Sanger sequencing method. This sequencing
25 reaction can be facilitated using DYEnamic™ ET* Terminator Cycle Sequencing Kits available from Amersham Biosciences (Piscataway; N.J.). Other suitable sequencing protocols include sequencing by synthesis protocols, such as those described in U.S. Patent Nos. 4,863,849, 5,405,746, 6,210,891, and 6,258,568; and PCT Applications Nos. WO 98/13523, WO 98/28440, WO 00/43540, WO 01/42496, WO 02/20836 and
30 WO 02/20837.

In addition to the primers and primer sets used for amplification of the HLA alleles, also provided are primers, primer sets and methods for sequencing HLA alleles following amplification. Generally, the methods for sequencing use the sequencing primers and primer sets. In some of these sequencing primers or methods, the 5' portion of one or more of the sequencing primers contains non-homologous or sequence that does not hybridize to the HLA allele but can provide enhanced resolution of the sequence generated early in the polymerization reaction. For example, by having or adding additional nonhomologous bases to the 5' end of the sequencing primer, the non-complementary portion can achieve enhanced resolution of sequence. It is believed, although not meant to be limiting, that this increased resolution occurs because the first bases resolved on any sequencing system are unclear. Clarity tends to improve within 30 to 35 bases from the 5' end of the sequencing primer as the time in the capillary of the sequencer is increased. Thus, the primer design encompassing additional non-homologous bases is particularly useful in sequencing primers that hybridize close to, for example within 10, 15, 20, 25, 30 or bases, of an intron/exon junction, such as where locus structure dictates placement of the primer close to the junction, such as that required with exons 2 and 3. The sequencing primer design is advantageous because many transplant clinics demand that the exons, such as exon 3, be covered completely with usable sequence. Where the exon sequence is very close to the 3' end of a sequencing primer, the sequence tends to be poorly resolved and valuable exonic data is lost during sequencing. In light of this, in certain embodiments of the invention, it is advantageous to place the sequencing primer far enough away from the intron/exon junction so that this near resolution is not an issue. Unfortunately, with some HLA loci, especially the class I loci, there are commonly insertion/deletion events near the intron/exon junctions. In some of these loci, depending on the allelic combination, sequencing primers cannot be placed upstream to an insertion/deletion because of resulting unreadable sequence. In these cases, it is preferential to anneal the primers near the junctions. As stated above, when the primers are near the intron/exon junctions, the addition of non-homologous bases to the primers provides additional sequence clarity. Generally, the number of the additional non-hybridizing bases added to the 5' end of the sequencing primers can vary as desired, and for example can be one to 35 bases, such as 2, three,

four, five, ten, fifteen, twenty, etc. An example of a typical HLA allele determination reaction used with the primers, primer sets and methods consists of the following reagents and reaction criteria:

Reagents

- 5 10X PCR buffer (brought down to between a 0.5X-2.0X concentration in the reaction (generally 1X is preferred))
- MgCl₂ (MgCl₂ in class I reactions typically ranges from 1.0mM – 2.0mM concentration in the reaction (generally 1.5mM is preferred); MgCl₂ in class II reactions typically ranges from 1.5mM – 2.5mM concentration in the reaction
- 10 (generally 2.0mM is preferred for single tube amplifications and 2.5mM is preferred for group specific amplifications)).
- dNTPs (brought to between a 0.5% - 2% concentration in the reaction (generally 1% is preferred)).
- DMSO (can be used at 5%-15% concentration in the reaction for class
- 15 I reactions (generally 8% is preferred)).
- Primers (concentration can vary and is successfully at ranges of 10 pmol/μl – 30 pmol/μl; optimal concentrations vary depending upon the reaction conditions, primer sequence and target sequence)

Reaction Criteria

- 20 Background should be less than 20% of overall signal.
- There should not be more than a 30% difference in the evenness of the peaks.
- Average signal strength should be between 100 and 4000 units when using a capillary (typically when signals get above 2000 – 2500 units, excessive
- 25 background will result); between 40 and 4000 units when using an ABI 377 automatic sequencer (generally signal will never get above about 500 units)
- The full sequence of the exon in question should be readable from beginning to end as a result of the sequencing reaction.

In some embodiments, HLA allele amplification reactions and HLA sequencing reactions can be joined together. The combination of HLA allele amplification and sequencing allows the resolution of many of the HLA alleles. Accordingly, in these embodiments, the present primer sets, methods and kits can
5 resolve greater than or about 50%, 55%, 60%, 65%, 70%, 75%, 80% or more of cis/trans ambiguities, including those found in the HLA B locus.

Although the present primers generally utilize the five standard nucleotides (A, C, G, T and U) in the nucleotide sequences, the identity of the nucleotides or nucleic acids used in the present invention are not so limited. Non-
10 standard nucleotides and nucleotide analogs, such as peptide nucleic acids and locked nucleic acids can be used in the present invention, as desired. In some embodiments, deazaG is used in order to increase the amplification of certain alleles that when in combination with other alleles will not amplify when all "natural" nucleotide primers are used. The addition of deazaG increases amplification of loci with high GC
15 percentages, such as what is found in many of the class I loci.

Nucleotide analogs are known in the art (e.g., see, Rawls, C & E News Jun. 2, 1997: 35; Brown, Molecular Biology LabFax, BIOS Scientific Publishers Limited; Information Press Ltd, Oxford, UK, 1991). When used with the primers, primer sets and methods of the present invention, these nucleotide analogs may include
20 any of the known base analogs of DNA and RNA such as, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, hypoxanthine, inosine, N6-isopentenyladenine, 1-methyladenine, 1-
25 methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid
30 methylester, uracil-5-oxyacetic acid, oxybutoxosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, orotic acid, 2,6-diaminopurine

and the AEGIS™ bases isoC and isoG. As such, the primers can contain DNA, RNA, analogs thereof or mixtures (chimeras) of these components. In addition to the use of non-standard nucleotides and nucleotide analogs, the bases in the primer sequences may be joined by a linkage other than a phosphodiester bond, such as the linkage bond
5 in a peptide nucleic acid, as long as the bond does not interfere with hybridization.

Universal nucleotides can also be used in the present primers. In some instances, nucleotide analogs and universal nucleotides will encompass the same molecules. As used herein, universal nucleotide, base, nucleoside or the like, refers to a molecule that can bind to two or more, i.e., 3, 4, or all 5, naturally occurring bases in a
10 relatively indiscriminate or non-preferential manner. In some embodiments, the universal base can bind to all of the naturally occurring bases in this manner, such as 2'-deoxyinosine (inosine). The universal base can also bind all of the naturally occurring bases with equal affinity, such as 3-nitropyrrole 2'-deoxynucleoside (3-nitropyrrole) and those disclosed in U.S. Patent Nos. 5,438,131 and 5,681,947. Generally, when the
15 base is "universal" for only a subset of the natural bases, that subset will generally either be purines (adenine or guanine) or pyrimidines (cytosine, thymine or uracil). An example of a nucleotide that can be considered universal for purines is known as the "K" base (N6-methoxy-2,6-diaminopurine), as discussed in Bergstrom *et al.*, Nucleic Acids Res. 25:1935 (1997). And an example of a nucleotide that can be considered
20 universal for pyrimidines is known as the "P" base (6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one), as discussed in Bergstrom *et al.*, *supra*, and U.S. Patent No. 6,313,286. Other suitable universal nucleotides include 5-nitroindole (5-nitroindole 2'-deoxynucleoside), 4-nitroindole (4-nitroindole 2'-deoxynucleoside), 6-nitroindole (6-nitroindole 2'-deoxynucleoside) or 2'-deoxynebularine. When universal nucleotides
25 are used, a partial order of base-pairing duplex stability has been found as follows: 5-nitroindole > 4-nitroindole > 6-nitroindole > 3-nitropyrrole. When used, such universal bases can be placed in one or more polymorphic positions, for example those that are not required to specifically identify an allele. Combinations of these universal bases at one or more points in the primers can also be used as desired. Primers and
30 strategies using universal primers are discussed in U.S. Patent Application Serial No. 10/429,912.

Moreover, any or all of the present primers or primer sets regardless of the HLA allele to which they hybridize can be labeled with a detectable moiety, if desired, to facilitate detection. When present, the detectable moiety of the present invention is not particularly limited. Suitable examples of detectable labels include
5 fluorescent molecules, beads, polymeric beads, fluorescent polymeric beads and molecular weight markers. Polymeric beads can be made of any suitable polymer including latex or polystyrene. One of skill in the art understands that any detectible label known in the art may be used with the primers and primer sets as long as the detectible label does not interfere with the primers, primer sets or methods of the
10 invention.

The present invention also provides arrays of the present primers that are contained within distinct, defined locations on a support. The skilled artisan understands that arrays can be used with the amplification and/or sequencing primers, primer sets and methods of the present invention. Any suitable support can be used
15 for the present arrays, such as glass or plastic, either of which can be treated or untreated to help bind, or prevent adhesion of, the primer. In some embodiments, the support will be a multi-well plate so that the primers need not be bound to the support and can be free in solution. Such arrays can be used for automated or high volume assays for target nucleic acid sequences.

20 In some embodiments, the primers will be attached to the support in a defined location. The primers can also be contained within a well of the support. Each defined, distinct area of the array will typically have a plurality of the same primers. As used herein the term "well" is used solely for convenience and is not intended to be limiting. For example, a well can include any structure that serves to
25 hold the nucleic acid primers in the defined, distinct area on the solid support. Non-limiting example of wells include depressions, grooves, walled surroundings and the like. In some of the arrays, primers at different locations can have the same probing regions or consist of the same molecule. This embodiment is useful when testing whether nucleic acids from variety of sources contain the same target sequences. The
30 arrays can also have primers having one or multiple different primer regions at different locations within the array. In these arrays, individual primers can recognize different alleles with different sequence combinations from the same positions, such

as for example with different haplotypes. This embodiment can be useful where nucleic acids from a single source are assayed for a variety of target sequences. In certain embodiments, combinations of these array configurations are provided such as where some of the primers in the defined locations contain the same primer regions, 5 whereas other defined locations contain primers with primer regions that are specific for individual targets.

The present invention also provides kits for carrying out the methods described herein. In one embodiment, the kit is made up of one or more of the described primers or primer sets with instructions for carrying out any of the methods 10 described herein. The instructions can be provided in any intelligible form through a tangible medium, such as printed on paper, computer readable media, or the like. A plurality of each primer or primer set can be provided in a separate container for easy aliquoting. The present kits can also include one or more reagents, buffers, hybridization media, salts, nucleic acids, controls, nucleotides, labels, molecular 15 weight markers, enzymes, solid supports, dyes, chromatography reagents and equipment and/or disposable lab equipment, such as multi-well plates (including 96 and 384 well plates), in order to readily facilitate implementation of the present methods. Such additional components can be packaged together or separately as desired. Solid supports can include beads and the like whereas molecular weight 20 markers can include conjugatable markers, for example biotin and streptavidin or the like. Enzymes that can be included in the present kits include DNA polymerases and the like. In some embodiments, kits include all reagents, primers, equipment etc. needed to perform the HLA amplification and/or sequencing except for the sample to be tested. Examples of kit components can be found in the description above and in 25 the following examples.

Exemplary, but non-limiting, primers and primer sets are described in Table 1. Sequence alignments for assigning positions can be obtained by comparing the listed sequences with reported HLA sequences which can be found at www.ebi.ac.uk/imgt/hla and www.anthonynolan.org.uk/research.html. In the reported 30 sequences, letter other than A, C, G or T indicate non-standard universal bases as follows: R, Y, S, M, W, and K are degenerate bases consisting of two possible bases at the same position. A or G = R, C or T = Y, G or C = S, C or A = M, A or T = W

and G or T = K. There are also combinations of 3 possible bases at a particular base position known as H, B, V. Although primer pairs are often used in nucleic acid amplifications, the present primer sets can contain odd numbers of primers so that one or more forward primers can work in conjunction with a single reverse primer to produce an amplicon and vice versa. Table 1 provides a list of exon identities and ambiguous typing combinations of some HLA alleles that can be resolved by the present primer sets, methods and kits. It is to be understood that any combination of the primers listed in Table 1 can be combined into a primer set. The only requirement is that the assembled primer set be capable of performing at least one step in one or more of the methods of the present invention. Table 1, in the primer sets labeled group specific or multiplex primers gives examples of primer sets that have been assembled. Each individual section of Table 1 demonstrates embodiments of primer sets of the present invention. The skilled artisan will understand that individual primers or combinations of primers that encompass less than the entire section of Table 1 may be used in alternative embodiments.

In Table 1, locations of hybridization for some of the primers are given. The skilled artisan will understand how to determine particular primer binding sites on HLA alleles of the primers where the specific location is not included in Table 1 using methods well-known in the art, such as BLAST sequence alignment.

TABLE 1

* All primers in Table 1 are written in the 5' to 3' direction

A Locus Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
pa5-3	HLA-A	amp primer	CAGACSCCGAGGATGGCC (SEQ ID NO.: 1)	0.5µl	20µM
pa3-29	HLA-A	amp primer	GCAGCGACCAACAGTCCAG (SEQ ID NO.: 2)	0.5µl	20µM
pa5-5	HLA-A	5' amp primer	ACCAGAACTCGCTGTTCCCTYYTCAGGGA (SEQ ID NO.: 3)	0.5µl	20µM
pa3-31	HLA-A	3' amp primer	AAAGTCACGGKCCCAAGGCTGCTGCCCKGTG (SEQ ID NO.: 4)	0.5µl	20µM
pa3-29-2	HLA-A	amp primer	TCACRGCAGCGACCAACAGTCCAG (SEQ ID NO.: 5)	0.5µl	20µM
A 3' UT	HLA-A		GCCTTTGCAGAAACAAAGTCAGGGTTC (SEQ ID NO.: 6)	0.5µl	20µM
	HLA-A	5' amp primer	CCCCAGACSCCGAGGATGGCC (SEQ ID NO.: 7)		
	HLA-A	3' amp primer	GGAAAGTCACGGKCCCAAGGCTGCTGCCCKGTG (SEQ ID NO.: 8)		
	HLA-A	5' amp primer	CTTGTTCTCTGCTTCCCACTCAATGTGTG (SEQ ID NO.: 9)		
	HLA-A	Ex4 amp primer	GCTGAGATCAGGTCCCATCACTGCCGTA (SEQ ID NO.: 10)		
	HLA-A	Ex4 amp primer	GCTGAGATCAGGTCCCATCACCGCTGTA (SEQ ID NO.: 11)		
	HLA-A	Ex4 amp primer	GCTGAGATCAGGTCCCATCACCGCCATA (SEQ ID NO.: 12)		
	HLA-A	Ex4 amp primer	GCTGAGATCAGGTCCCATCACCGCCGTA (SEQ ID NO.: 13)		
	HLA-A	seq primer	GGGAAACSGCCTCTG (SEQ ID NO.: 14)		
Aex2R-4	HLA-A	seq primer	GGATCTCGGACCCGGAGACTGT (SEQ ID NO.: 15)		
Aex3F-2	HLA-A	seq primer	CCCGGTTTCA TTTTCAGTTTAGG (SEQ ID NO.: 16)		
Aex3R-3	HLA-A	seq primer	ATTCTAGTGTGGTCCCAATTGTCTC (SEQ ID NO.: 17)		
Aex4F	HLA-A	seq primer	GGTGTCTGTCCATTCTC (SEQ ID NO.: 18)		
	HLA-A	seq primer	GAGAGGCTCCTGCTTCCCTA (SEQ ID NO.: 19)		
Aex2F-2	HLA-A	seq primer	GCCTCTGYGGGGAGAAGCAA (SEQ ID NO.: 20)	1µl	3µM
Aex4R-4	HLA-A	seq primer	CAGAGAGGCTCCTGCTTTC (SEQ ID NO.: 160)	1µl	3µM

A Locus Multiplex Amplification Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
pa5-3	HLA-A	5' amp primer	CAGACSCCGAGGATGGCC (SEQ ID NO.: 1)		
pa5-5	HLA-A	5' amp primer	ACCAGAACTCGCTGTCCCTYTCAGGGA (SEQ ID NO.: 3)		
pa3-31	HLA-A	3' amp primer	AAAGTCACGGKCCCAAGGCTGCTGCKGTG (SEQ ID NO.: 4)		
pa3-29-2	HLA-A	3' amp primer	TCACRGCAGCGACCAACAGCTCCAG (SEQ ID NO.: 5)		

B Locus Primers

Primer ID	Locus	Primer Type	Primer Sequence	Location	Amount/ rxn	Final Molarity
pB3-24	HLA-B	3' amp primer	GGTKCCCCAAGGCTGCTGCAGGGG (SEQ ID NO.: 21)	intron 3, bases 1234-1256	0.5µl	20µM
pB5-48	HLA-B	amp primer	GAACCGTCTCTCTGCTGCTCTC (SEQ ID NO.: 22)	exon 1 bases 20-41	0.5µl	20µM
pB5-49	HLA-B	amp primer	GAACCGTCTCTCTGCTGCTCTG (SEQ ID NO.: 23)	exon 1 bases 20-41	0.5µl	20µM
pB3-20	HLA-B rev	3' amp primer	ATCACAGCAGCGACCAACAGCTCCGAT (SEQ ID NO.: 24)	exon 5, bases 968-994	0.5µl	10µM
pB3-21	HLA-B rev	3' amp primer	ATCACAGTAGCGACCAACAGCTCCGAT (SEQ ID NO.: 25)	exon 5, bases 968-994	0.5µl	10µM
pB3-22	HLA-B rev	3' amp primer	ATCACAGTAGCAACCAACAGCTCCGAT (SEQ ID NO.: 26)	exon 5, bases 968-994	0.5µl	10µM
pB3-23	HLA-B rev	3' amp primer	ATCACAGCAGCGACCAACAGCGACCCAC (SEQ ID NO.: 27)	exon 5, bases 968-994	0.5µl	10µM
pB5-55+4	HLA-B	5' amp primer	GGCTCTGATTCCAGCACTTCTGAGTCACTTTAC (SEQ ID NO.: 28)	intron 3, bases 1308-1340	0.5µl	20µM
pB5-52	HLA-B	5' amp primer	GACCAACAGGCTGGGGCGCAGGACCCGG (SEQ ID NO.: 29)	intron 1, bases 122-148	0.5µl	20µM
pB5-53	HLA-B	5' amp primer	GACCAACAGGCGGGGGCGCAGGACCTGA (SEQ ID NO.: 30)	intron 1, bases 122-148	0.5µl	20µM
pB5-44	HLA-B	5' amp primer	ACGCACCCACCCCGGACTCAGAA (SEQ ID NO.: 31)	5' untranslated region, bases -39 to -18	0.5µl	20µM
pB5-45	HLA-B	5' amp primer	ACGCACCCACCCCGGACTCAGAG (SEQ ID NO.: 32)	5' untranslated region, bases -39 to -18	0.5µl	20µM

Primer ID	Locus	Primer Type	Primer Sequence	Location	Amount/ rxn	Final Molarity
B 3' UT	HLA-B	3' amp primer	AGAGGCTCTTTGAAGTCACAAAGGGGA (SEQ ID NO.: 33)	3' untranslated region, bases 2913-2938	0.5µl	20µM
yB2F- 6a+10	HLA-B	5' amp primer	ACTGTGAACCGTCCTCCTGCTGCTCTC (SEQ ID NO.: 34)	intron 1, bases 189-201 + nonsense bases	1µl	3µM
	HLA-B	5' amp primer	AAGTGCGAACCCTCCTCCTGCTGCTCTG (SEQ ID NO.: 35)			
	HLA-B	5' amp primer	AAGTGCGAACCCTCCTCCTGCTGCTCTG (SEQ ID NO.: 36)			
	HLA-B	3' amp primer	ACTGCGGTKCCCAAGGCTGCTGCAGGGG (SEQ ID NO.: 37)			
	HLA-B	seq primer	ATTATGATTAAAGCCCTCCTCRCCCCAG (SEQ ID NO.: 38)			
yB2R-4	HLA-B	seq primer	ATTATGATTACAGCCCTCCTTGCCCCAG (SEQ ID NO.: 39)	intron 3, bases 1100-1121 intron 3, bases 1494-1511 intron 4, bases 1871-1891 intron 1, bases 189-201 +nonsense bases	1µl	3µM
	HLA-B	seq primer	ATTATGATTAAAGCCCTCCTTGCCCCAG (SEQ ID NO.: 40)			
	HLA-B	seq primer	GGAGGGTCTGACCTGCG (SEQ ID NO.: 41)			
	HLA-B	seq primer	ATTATGATTAGGGGACGGGCTGACC (SEQ ID NO.: 42)			
	HLA-B	seq primer	ATTATGATTAGGGGACTGGCTGACC (SEQ ID NO.: 43)			
	HLA-B	seq primer	ATTATGATTAGGGGACGGTCTGACC (SEQ ID NO.: 44)			
	HLA-B	seq primer	AAACTCATGCCATTCTCCATT (SEQ ID NO.: 45)			
	HLA-B	seq primer	GTCACATGGTGGTCTTA (SEQ ID NO.: 46)			
	HLA-B	seq primer	GGCTCCTGCTTTCCCTGAGAA (SEQ ID NO.: 47)			
	HLA-B	seq primer	ATTATGATTAAAGCCCTCCTCRCCCCAG (SEQ ID NO.: 38)			
B-Ex3R	HLA-B	seq primer	CAGCCCTCCTTGCCCCAG (SEQ ID NO.: 48)	intron 1, bases 189-201) intron 1, bases 189-201) intron 1, bases 189-201) intron 1, bases 189-201) intron 1, bases 189-201)		
B-Ex4F1	HLA-B	seq primer	AGCCCTCCTCRCCCCAG (SEQ ID NO.: 49)			
yB4R-3	HLA-B	seq primer	AGCTCCTCCTCGCCCCAG (SEQ ID NO.: 50)			
yB2F- 6a+10	HLA-B	seq primer	AGCCCTCCTGCCCCAG (SEQ ID NO.: 51)			
yB2F-5a	HLA-B	seq primer	GGGACGGGGTACC (SEQ ID NO.: 52)			
yB2F-6a	HLA-B	seq primer	GGGACTGGGTACC (SEQ ID NO.: 53)			
yB2F-7a	HLA-B	seq primer	GGGACGGGTGCTGACC (SEQ ID NO.: 54)			
yB2F-12a	HLA-B	seq primer				
yB3F-2a	HLA-B	seq primer				
yB3F-2b	HLA-B	seq primer				
yB3F-2c	HLA-B	seq primer				

Atty. Dkt. No.: 044487-0147

B Locus Multiplex Amplification Primers

Primer ID	Locus	Primer Type	Primer Sequence	Location	Amount/ rxn	Final Molarity
pB5-48	HLA-B	5' amp primer	GAAACCGTCCTCCTGCTGCTCTC (SEQ ID NO.: 22)	exon 1 bases 20-41		
pB5-49	HLA-B	5' amp primer	GAAACCGTCCTCCTGCTGCTCTG (SEQ ID NO.: 23)	exon 1 bases 20-41		
pB3-20	HLA-B	3' amp primer	ATCACAGCAGCGACCGACCAAGCTCCGAT (SEQ ID NO.: 24)	exon 5, bases 968-994		
pB3-21	HLA-B	3' amp primer	ATCACAGTAGCGACCGACCAAGCTCCGAT (SEQ ID NO.: 25)	exon 5, bases 968-994		
pB3-22	HLA-B	3' amp primer	ATCACAGTAGCAACCAAGCTCCGAT (SEQ ID NO.: 26)	exon 5, bases 968-994		
pB3-23	HLA-B	3' amp primer	ATCACAGCAGCGACCGACCAAGCTCCGAT (SEQ ID NO.: 27)	exon 5, bases 968-994		
pB5-55+4	HLA-B	5' amp primer	GGCTCTGATTCCAGGCACTTCTGAGTCACTTTAC (SEQ ID NO.: 28)	intron 3, bases 1308-1340		
pB3-24	HLA-B	3' amp primer	GGTKCCCCAAGGCTGCTGCAGGGG (SEQ ID NO.: 21)	intron 3, bases 1234-1256		

C Locus Single Tube Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
C Intron 3 R	HLA-C		GCAGTGGTCAAAAGTGGTCA (SEQ ID NO.: 55)		
C Intron 3 F	HLA-C		GCAGCTGTGGTCAGGGTCTGCT (SEQ ID NO.: 56)		
C 3' UT	HLA-C		GGACACGGGGGTGRGCTGTCTSTC (SEQ ID NO.: 57)		
C5ApUTG	HLA-C		CAGTCCCGGTTCTGAAGTCCCCAGT (SEQ ID NO.: 58)		
C5ApUTA	HLA-C		CAGTCCCGGTTCTAAAGTCCCCAGT (SEQ ID NO.: 59)		
C5X1_I1GG	HLA-C		GGGCGGTGAGTGGGGGTT (SEQ ID NO.: 60)		
C5X1_I1TA	HLA-C		GGGCGTGTGAGTGCAGGGTT (SEQ ID NO.: 61)		
C5X1_I1TG	HLA-C		GGGCGTGTGAGTGGGGGTT (SEQ ID NO.: 62)		
C3ApX5A	HLA-C		AGTCCAAAGGACAGCTAGGACA (SEQ ID NO.: 63)		
C3ApX5T	HLA-C		AGTCTTAGGACAGCTAGGACA (SEQ ID NO.: 64)		
C173ApX5	HLA-C		GACAGCCAGGACAGCCAGGACA (SEQ ID NO.: 65)		
C3ApI4T	HLA-C		GTGAGGGGCCCTGACCTCCAA (SEQ ID NO.: 66)		
C3ApI4C	HLA-C		GTGAGGGGCCCTGACCCCAA (SEQ ID NO.: 67)		
C3ApI4TAC	HLA-C		GTGAGGGGCCCTTACACCCAA (SEQ ID NO.: 68)		
CpExon5R2	HLA-C		GCCATCACAGCTCCTAGGACAGCTA (SEQ ID NO.: 69)		
CpExon5R3	HLA-C		GCCACCATAGCTCCTAGGACAGCTA (SEQ ID NO.: 70)		
CpExon5R4	HLA-C		GTGACCCACAGCTCCAAAGGACAGCTA (SEQ ID NO.: 71)		
CpExon5R5	HLA-C		AGTAGGACAGCCAGGACAGCCA (SEQ ID NO.: 72)		
CpExon5R1	HLA-C		CCACCACAGCTCCTAGGACAGCTA (SEQ ID NO.: 73)		
pC5-2	HLA-C		CAGTCCCGGTTCTRAAGTCCCCAGT (SEQ ID NO.: 74)		
C5x21	HLA-C		GGAGCCGCGCAGGGAGG (SEQ ID NO.: 75)		
c5x22	HLA-C		GGGTCCGGCGGCTCTCAG (SEQ ID NO.: 76)		
c3x21	HLA-C		GGCCGTCCGTGGGGATG (SEQ ID NO.: 77)		
c3x22	HLA-C		TCGKGACCTGCGCCCG (SEQ ID NO.: 78)		
c5x31	HLA-C		TTTCRGTTTAGGCCAAAATCCCCG (SEQ ID NO.: 79)		
c5x32	HLA-C		GTCCCTTTACCCGGTTTCA TTTTC (SEQ ID NO.: 80)		

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Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
c3x31	HLA-C		GCTGATCCCAATTTCTCCCTCC (SEQ ID NO.: 81)		
c5x41	HLA-C		AGGTGGCGTCTGGGTCTGTG (SEQ ID NO.: 82)		
c5x42	HLA-C		CCRTTCTCAGGATRGTCACATGGC (SEQ ID NO.: 83)		
c5x43	HLA-C		CAAGTGCTGAAATTTCTGACTCTTCCC (SEQ ID NO.: 84)		
c3x41	HLA-C		AGGACTTCTGCTTTCYCTGAKAAG (SEQ ID NO.: 85)		
	HLA-C	amp primer	CCACTCCCATTTGGGTGCGGRTTCT (SEQ ID NO.: 86)		
	HLA-C	amp primer	CCACAGCTGCYGCAGTAGTCAAAAGTGGTC (SEQ ID NO.: 87)		
	HLA-C	amp primer	CTCAGGTCAGGACCAGAAAGTCGCTGTTCAT (SEQ ID NO.: 88)		
	HLA-C	amp primer	CTGAGATGGCCCCAGGTGTGGATGG (SEQ ID NO.: 89)		
	HLA-C	amp primer	CTGAGATGGCCCCATGTGTGGATGG (SEQ ID NO.: 90)		
	HLA-C	seq primer	ATGATATTATGATTAGGAGCCCGCGCAGGGAGG (SEQ ID NO.: 91)		
	HLA-C	seq primer	ATTATGATTACTCGGGGGACGGGGCTGACC (SEQ ID NO.: 92)		
	HLA-C	seq primer	ATGATTAAACCCCTCATCCCCCTCCTTA (SEQ ID NO.: 93)		
	HLA-C	seq primer	ATGATTAAACCCCCCAATCCCCCTCCTTA (SEQ ID NO.: 94)		

DR Locus Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
OTDR-01	DRB1	5' amp primer	TGTAAACGACGGCCAGTCCACAGCACGTTTCTTGTG (SEQ ID NO.: 95)		
OTDR-02/07	DRB1	5' amp primer	TGTAAACGACGGCCAGTCCACAGCACGTTTCTGT (SEQ ID NO.: 96)		
OTDR-03/5/6/08/12	DRB1	5' amp primer	TGTAAACGACGGCCAGTTTCACAGCACGTTTCTTGGAGTAC (SEQ ID NO.: 97)		
OTDR-04	DRB1	5' amp primer	TGTAAACGACGGCCAGTTACTAATCACGTTTCTTGGAGCAGGT (SEQ ID NO.: 98)		
OTDR-09	DRB1	5' amp primer	TGTAAACGACGGCCAGTTCCACAGCACGTTTCTTGA (SEQ ID NO.: 99)		
OTDR-10	DRB1	5' amp primer	TGTAAACGACGGCCAGTTACTAATCACGTTTCTTGGAGGAGG (SEQ ID NO.: 100)		
	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTTACTAATCACGTTTCTTGGAGCAGGTTAAAC (SEQ ID NO.: 126)		
	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTATCACAGCACGTTTCTTGGAGG (SEQ ID NO.: 127)		
	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTTACTAATCACGTTTCTTGAAGCAGGATAAGTT (SEQ ID NO.: 128)		
	HLA-DR	3' amp primer	CAGGAAACAGCTATGACCCRYGCTYACCTCGCKCTG (SEQ ID NO.: 129)		
M13 Forward		seq primer	TGTAAACGACGGCCAGT (SEQ ID NO.: 116)		
M13 Reverse		seq primer	CAGGAAACAGCTATGACC (SEQ ID NO.: 117)		

DR Locus Group Specific Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
GSDR-01	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCACGTTTCTTGTGGSAGCTT (SEQ ID NO.: 101)		

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Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
GSDR-15/16	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTTTCCTGTGGCAGCCTAAGA (SEQ ID NO.: 102)		
GSDR-03/11/13/14	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCGTTTCTTGGAGTACTCTACGTC (SEQ ID NO.: 103)		
GSDR-04	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCGTTTCTTGGAGCAGGTTAAAC (SEQ ID NO.: 104)		
GSDR-07	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTTTCCTGTGGCAGGGTAAGTATA (SEQ ID NO.: 105)		
GSDR-08/12	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCGTTTCTTGGAGTACTCTABGGG (SEQ ID NO.: 106)		
GRDR-09	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTGTTCCTTGAAGCAGGATAAGTT (SEQ ID NO.: 107)		
GSDR-10	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCACAGCAGCGTTTCTTGGAGG (SEQ ID NO.: 108)		
GSDR-B3	HLA-DR	5' amp primer	ACAGCTCCAGYAWCACYAG (SEQ ID NO.: 109)		
GSDR-B4	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTAGCGAGTGTGGAACCTGATC (SEQ ID NO.: 110)		
GSDR-B5	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTCGACGACGAGGATAAGTATGA (SEQ ID NO.: 111)		
GSDR-3' Universal	HLA-DR	3' amp primer	CAGGAACAGCTATGACCGCTYACCTCGCCCKCTGCAC (SEQ ID NO.: 112)		
CRP 1	HLA-DR	5' amp primer	TCATGCTTTTGGCCAGACAG (SEQ ID NO.: 113)		
CRP 3	HLA-DR	3' amp primer	GGCGGACTCCAGCTTGTGA (SEQ ID NO.: 114)		
	HLA-DR	5' amp primer	TGTAAACGACGGCCAGTGSAGCTGYKTAAGTCTGAGT (SEQ ID NO.: 115)		
M13 Forward		seq primer	TGTAAACGACGGCCAGT (SEQ ID NO.: 116)		
M13 Reverse		seq primer	CAGGAACAGCTATGACC (SEQ ID NO.: 117)		
	Codon86-GTG	seq primer	CTGCACYGTGAACKCTCTCCA (SEQ ID NO.: 118)		

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DR Locus Group Specific and Single Tube Sequencing Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
M13 Forward		seq primer	TGTAAACGACGGCCAGT (SEQ ID NO.: 116)		
M13 Reverse		seq primer	CAGGAAACAGCTATGACC (SEQ ID NO.: 117)		

DR Locus Group Specific Sequencing Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
	HLA-DR	seq primer	CTGTGGCAGGTAAGTATA (SEQ ID NO.: 119)		
	HLA-DR	seq primer	TTCTTGGAGCAGGTTAAAC (SEQ ID NO.: 120)		
	HLA-DR	seq primer	CCTGTGGCAGCCTAAGA (SEQ ID NO.: 121)		
	HLA-DR	seq primer	CGTTTCTTGTGGSAGCTT (SEQ ID NO.: 122)		
	HLA-DR	seq primer	TTCTTGGAGTACTCTACGTC (SEQ ID NO.: 123)		
	HLA-DR	seq primer	CCACAGCACGTTTCTTGTG (SEQ ID NO.: 124)		
	HLA-DR	seq primer	CGTTTCTTGGAGTACTCTACGGG (SEQ ID NO.: 125)		

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DP Locus Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
	HLA-DP	amp primer		5' to 3' sequence of DPB primer	
	HLA-DP	amp primer	TGTAAACGACGGCCAGTCCTCCCCGCAGAGAAATTAMGTG (SEQ ID NO.: 130)		
	HLA-DP	amp primer	TGTAAACGACGGCCAGTCCTCCCCGCAGAGAAATTACCTT (SEQ ID NO.: 131)		
	HLA-DP	amp primer	CAGGAAACAGCTATGACCGCGCTGYAGGGTCACGGCCT (SEQ ID NO.: 132)		
	HLA-DP	amp primer	CAGGAAACAGCTATGACCGCGCTGCAGGGTCA TGGGGCC (SEQ ID NO.: 133)		
CRP1	HLA-DP	seq primer	TCA TGCTTTTGGCCAGACAG (SEQ ID NO.: 113)		
CRP3	HLA-DP	seq primer	GGCGGACTCCCAGCTTGTA (SEQ ID NO.: 114)		
M13 Forward		seq primer	TGTAAACGACGGCCAGT (SEQ ID NO.: 116)		
M13 Reverse		seq primer	CAGGAAACAGCTATGACC (SEQ ID NO.: 117)		

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DQ Locus Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
M13 Forward	HLA-DQ	amp primer	TGTAACACGACGGCCAGTGGTGATTCCCCCGCAGAGGAT (SEQ ID NO.: 134)		
	HLA-DQ	amp primer	CAGGAACAGCTATGACCCGGGCTCGCAGASGGGCGACG (SEQ ID NO.: 135)		
	HLA-DQ	amp primer	CAGGAACAGCTATGACCCGGGCTCACGGAGGGGCGACG (SEQ ID NO.: 136)		
	HLA-DQ	amp primer	CAGGAACAGCTATGACCCGGGCTCACGGAGGGGTCAACC (SEQ ID NO.: 137)		
M13 Reverse		seq primer	TGTAACACGACGGCCAGT (SEQ ID NO.: 116)		
		seq primer	CAGGAACAGCTATGACC (SEQ ID NO.: 117)		

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DQ Locus Multiplex Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
DQX3 Forward Amp DQX3 Reverse Amp 1 DQX3 Reverse Amp 2	HLA-DQ	amp primer	GTAAACGACGCCAGTGGCTGCTTGTGAGCAGAAG (SEQ ID NO.: 138)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTGTGCTACTTCACCAACGGGAGG (SEQ ID NO.: 139)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTGTGCTACTTCACCAACGGGAGC (SEQ ID NO.: 140)		
	HLA-DQ	amp primer	CAGGAAACAGCTATGACCTCGCCGCTGCAAGGTCGT (SEQ ID NO.: 141)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTGATTTTCGTGTACCAAGTTTAAGGGTC (SEQ ID NO.: 142)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTAGGATTTTCGTGTACCAAGTTTAAGGGTA (SEQ ID NO.: 143)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTAGGATTTTCGTGTTCAGTTTAAGGGTA (SEQ ID NO.: 144)		
	HLA-DQ	amp primer	GTAAACGACGCCAGTAGGATTTTCGTGTTCAGTTTAAGGGTA (SEQ ID NO.: 145)		
	HLA-DQ	amp primer	CAGGAAACAGCTATGACCTCTCCTCTGCAAGATCCC (SEQ ID NO.: 146)		
	HLA-DQ	amp primer	CAGGAAACAGCTATGACCTCTCCTCTGCAAGATCCC (SEQ ID NO.: 147)		
	HLA-DQ	amp primer	CAGTCGAGGCTGATAGCGAGCTCCCTGTCTGTACTGCCCTYAG (SEQ ID NO.: 148)		
	HLA-DQ	amp primer	CTATCAACAGGTTGAACTGGGCCCCACAGTAACAGAAACTCAATA (SEQ ID NO.: 149)		
	HLA-DQ	amp primer	CTATCAACAGGTTGAACTGGGCCCCATAATAACAGAAACTCAATA (SEQ ID NO.: 150)		

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Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
Reverse Seq	HLA-DQ	seq primer	CTATCAACAGGTTGAACTG (SEQ ID NO.: 151)		
Forward Seq	HLA-DQ	seq primer	CAGTCGAGGCTGATAGCGAGCT (SEQ ID NO.: 152)		
Primer					
M13 Forward		seq primer	TGTAAAAACGACGGCCAGT (SEQ ID NO.: 116)		
M13 Reverse		seq primer	CAGGAAACACAGCTATGACC (SEQ ID NO.: 117)		

DQ Locus Potential Group Sequencing Primers

Primer ID	Locus	Primer Type	Primer Sequence	Amount/ rxn	Final Molarity
	HLA-DQ	seq primer	GTGCGTCTTGTGAGCAGAAG (SEQ ID NO.: 153)		
	HLA-DQ	seq primer	GCTACTTCACCAACGGGAGG (SEQ ID NO.: 154)		
	HLA-DQ	seq primer	GCTACTTCACCAACGGGAGC (SEQ ID NO.: 155)		
	HLA-DQ	seq primer	TTCGTGTAACCAAGTTAAAGGTC (SEQ ID NO.: 156)		
	HLA-DQ	seq primer	ATTTCGTGTACCAAGTTAAAGGTA (SEQ ID NO.: 157)		
	HLA-DQ	seq primer	ATTTCGTGTCCAGTTAAAGGTA (SEQ ID NO.: 158)		
	HLA-DQ	seq primer	ATTTCGTGTCCAGTTAAAGGCTA (SEQ ID NO.: 159)		

EXAMPLES

Example 1 - An embodiment of a kit according to the present invention that contains primers capable of amplifying and sequencing HLA A locus, B locus and DRB1 locus alleles.

5 A Locus Single Tube

Amplification Primers: The single 5' primer begins in the A Locus 5' untranslated region and ends in exon 1. The single 3' primer is in exon 5. This is a locus specific amplification and all alleles in the A locus are amplified with this primer set.

10 *Sequencing Primers:* All sequencing primers are located in the introns flanking exons 2, 3 and 4.

B Locus Single Tube

15 *Amplification Primers:* Two 5' primers in exon 1, a C primer and a G primer. The alleles are segregated by the presence of a G or C at a defined base in exon 1. Approximately half of the alleles have a C at that position, the other half a G. The alleles in the B Locus, which are labeled according to convention known in the art are divided roughly in half between the two primers in exon 1 as follows in Table 2:

TABLE 2

C Group B Locus Alleles		G Group B Locus Alleles		
070201	380201	1301	4002	5611
070202	390101	1302	4003	570101
0703	390103	1303	4004	5702
0704	390201	1304	4005	570301
0706	390202	1308	400601	5706
0709	3903	180101	400602	5801
0718	3904	1802	4008	5802
0801	3905	1803	4013	5804
0802	390601	1806	4020	5901
1401	390602	2702	44020101	7801
1402	3908	2703	44020102S	780201
1405	3909	2704	44301	8101
15010101	3910	270502	440302	8202
1502	3917	270504	4404	8301
1503	3924	270505	406	
1508	400101	2706	4407	
1509	400102	2708	4408	
1510	4007	2709	4409	
151101	4012	2711	4413	
151102	4016	2712	4431	
1512	4023	2713	47010101	
1513	4101	2714	47010102	
1514	4102	2718	4702	
1515	4201	350101	510101	
1516	4418	3502	510102	
151701	4501	3503	510105	
151702	4504	3504	510201	
1518	4601	3505	510202	
1519	4801	3506	5103	
1520	4802	3507	5104	
1521	4805	3508	5108	
1523	4901	3511	520101	
1525	5001	3512	520102	
1528	5002	3515	5204	
1529	670101	3528	5301	
1546	6702	3531	5401	
1552	7301	3541	5501	
1553		3542	5502	
1554		3543	5505	
1555		3701	5512	
1557		3702	5601	
1558		3704	5602	
1566		3705	5603	

There are four 3' primers in exon 5 (primers are multiplexed to cover the complexity of B Locus in this exon). Thus, these primers anneal to four distinct sequences. In order to amplify all of the known alleles in HLA Locus B, each of the four primers was included in a cocktail of reverse primers.

5 *Sequencing Primers:* All sequencing primers are located in the introns flanking exons 2, 3 and 4.

DRB1 Single Tube

Amplification Primers: There are six 5' amplification primers that begin in intron 1 and end in exon 2. Each individual primers is designed to amplify a
10 specific group of alleles: DRB1*01, DRB1*15/16/07, DRB1*03/11/13/14/8/12, DRB1*04, DRB1*09, and DRB1*10. There is one 3' primer located in exon 2. All amplification primers are tailed with the M13 sequence. M13 sequence are tails, which do not bind to the HLA allele, that are added to the amplification primers, such as in DR, DQ, and DP that allow the utilization of a single forward and reverse primer
15 during a sequencing reaction irrespective of groups. This results in a reduction in the total number of sequencing primers that must be included in the kit to cover all possible products. The tailing of the amplification primers was also done to increase the resolution and assure full coverage of exon 2 upon sequencing.

Sequencing primers: The sequencing primers are M13 forward and
20 M13 reverse.

DRB1/3/4/5 Group Specific

Amplification primers: The primers either begin in intron 1 and end in exon 2 or are fully in exon 2 depending on where the most group specificity exists for the HLA alleles being amplified. There are eleven 5' group specific primers
25 amplifying the following groups/beta chains: DRB1*01, DRB1*15/16, DRB1*03/11/13/14, DRB1*04, DRB1*07, DRB1*8/12, DRB1*09, DRB1*10, DRB3, DRB4, DRB5. There is one 3' primer located in exon 2. All amplification

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primers are tailed with the M13 sequence. The tailing of the amplification primers was done to assure full coverage of exon 2 upon sequencing.

Sequencing primers: The sequencing primers are M13 forward and M13 reverse.

5

Example 2 - A and B Locus Multiplex Amplification

Genomic DNA was amplified with the following amplification mix:

A Locus

<i>Reagent</i>	<i>Amount</i>
Purified water	9.3µl
10X PCR Buffer	2.5µl
Magnesium Chloride	1.5µl
DMSO	2.0µl
dNTP (50% deazaG)	2.5µl
5'Primer- pA5-5	0.5µl
3'Primer- pA3-31	0.5µl
5'Primer- pA5-3	0.5µl
3'Primer- pA3-29-2	0.5µl
FastStart Taq	0.2µl
Genomic DNA	5.0µl
<hr/>	
25µl total reaction volume	

B Locus

<i>Reagent</i>	<i>Amount</i>
Purified water	9.3µl
10X PCR Buffer	2.5µl
Magnesium Chloride	1.5µl
DMSO	2.0µl
dNTP (50% deazaG)	2.5µl
5'Primer- pB5-48 or 5-49	0.5µl
3'Primer- pB3-24	0.5µl
5'Primer- pB5-55+4	0.5µl
3'Primer- pA3-20,21,22,23	0.5µl
FastStart Taq	0.2µl
Genomic DNA	5.0µl
<hr/>	
25ul total reaction volume	

Both A locus and B locus samples were run in a PE 9700 thermal cycler under the following conditions:

Initial Denaturation	95°C	4 min	} 35 cycles
Denaturation	95°C	20 sec	
Annealing	63°C	20 sec	
Extension	72°C	40 sec	
Final Extension	72°C	5 min	

Following amplification, the PCR amplicons were run on a 1.5% agarose gel to check for successful amplification. The results of the A locus agarose gel are demonstrated in Fig. 1A. For the A Locus, the ~1300bp band is the product of the amplification using pA5-3 and pA3-31 as the primers and the smaller ~700bp band is the product of the amplification using pA5-5 and pA3-29-2 as primers. The results of the B locus agarose gel are demonstrated in Fig. 1B. For the B Locus, the ~1250bp band is the product of the amplification using pB5-48 or pB5-49 and pB3-24 as primers and the smaller ~720bp band is the product of the amplification using pB5-55+4 and pB3-20, pB3-22, and pB3-23 as primers. In many cases, because the size of the amplicons is so similar between the loci, because the position of the primers on the HLA locus is similar, agarose gel electrophoresis will be used only to check the amplification reaction and not to distinguish between alternative HLA loci. However, in some instances, more sensitive techniques, such as using microfluidic separation may be used to distinguish HLA loci prior to sequencing.

Following confirmation of amplification, to prepare the amplicon for the sequencing reaction, 4µl of ExoSAP-IT® (USB; Cleveland, OH) was added to each amplicon to rid each amplicon of excess primer and dNTPs. Subsequent to the addition of the ExoSAP-IT®, the amplicons were incubated at 37°C for 20 minutes and then at 80°C for 20 minutes.

The next step was sequencing of the amplicons. Sequencing reactions for exons 2, 3 and 4 for both HLA A locus and HLA B locus were prepared for each sample using the following mix of reagents:

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DYEnamic™ ET Terminators (Amersham Biosciences)	2μl
DYEnamic™ ET Terminator Dilution Buffer	2μl
Water	3μl
Sequencing Primer (either forward or reverse)	1μl
ExoSAP-IT® treated PCR product	2μl
	<hr/>
	10μl total reaction volume

In order to gain sequence analysis, the entire reaction volume of the sequencing reactions were cycled in a PE 9700 thermal cycler under the following conditions:

95°C	20 sec	} 25 cycles
50°C	15 sec	
60°C	60 sec	
4°C	Infinite	

- Following completion of the sequencing reaction, ethanol precipitation
- 5 was used to remove excess terminators and precipitate out the sequencing products. The precipitated products were run on an ABI 3100 capillary sequencer. The electropherogram results of the sequencings reactions are shown in FIGS. 2A-2D.

- The present primers and kits can have any or all of the components described herein. Likewise, the present methods can be carried out by performing any
- 10 of the steps described herein, either alone or in various combinations. One skilled in the art will recognize that all embodiments of the present invention are capable of use with all other appropriate embodiments of the invention described herein. Additionally, one skilled in the art will realize that the present invention also encompasses variations of the present primers, configurations and methods that specifically exclude one or more
- 15 of the components or steps described herein.

- As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any
- 20 listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down

into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," "more than" and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. In the same manner, all
5 ratios disclosed herein also include all subratios falling within the broader ratio.

One skilled in the art will also readily recognize that where members are grouped together in a common manner, such as in a Markush group, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group. Accordingly, for
10 all purposes, the present invention encompasses not only the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the invention.

All references, patents and publications disclosed herein are
15 specifically incorporated by reference thereto. Unless otherwise specified, "a" or "an" means "one or more".

While preferred embodiments have been illustrated and described, it should be understood that changes and modifications can be made therein in accordance with ordinary skill in the art without departing from the invention in its broader aspects
20 as described herein.

CLAIMS

What is claimed is:

- 1 1. A primer set comprising:
2 primers that are capable of amplifying all human leukocyte antigen
3 (HLA) alleles of an HLA locus; and
4 a control primer pair that will produce an HLA control amplicon of
5 predetermined size from the one or more HLA alleles of a single HLA locus if the one
6 or more HLA alleles are present in a sample.
- 1 2. The primer set of claim 1 wherein the portion of the HLA allele
2 amplified by the control primer pair is common to all or substantially all HLA alleles.
- 1 3. The primer set of claim 1 wherein the portion of the HLA allele
2 amplified by the control primer pair comprises a portion of exon 4 of the HLA A
3 locus or HLA B locus.
- 1 4. The primer set of claim 1 wherein the HLA control amplicon is
2 about 500 to 1000 base pairs in length.
- 1 5. The primer set of claim 1 wherein the one or more HLA alleles
2 are common to a single HLA locus.
- 1 6. A primer set comprising:
2 a mixture comprising a plurality of primers that are capable of
3 simultaneously amplifying a plurality of a portion of class I HLA alleles
4 simultaneously.
- 1 7. The primer set of claim 6 wherein the plurality of Class I HLA
2 alleles belong to a same HLA locus.
- 1 8. The primer set of claim 7 wherein the HLA locus is HLA A
2 or HLA B.

1 9. The primer set of claim 6 wherein the plurality of primers are
2 capable of producing two amplicons from the HLA locus, and further wherein the two
3 amplicons spanning all HLA locus alleles.

1 10. The primer set of claim 9 wherein the first amplicon spans exon
2 1 to intron 3 and the second amplicon spans intron 3 to exon 5.

1 11. A primer for sequencing an HLA allele comprising:
2 a forward primer comprising a 3' portion and a 5' portion wherein the
3 3' portion is complementary to an HLA allele of interest and the 5' portion is not
4 complementary to the HLA allele of interest wherein the primer allows complete
5 resolution of an exonic sequence.

1 12. The primer of claim 11 wherein the 5' portion non-
2 complementary portion is 1 to about 35 bases inclusive.

1 13. The primer of claim 11 wherein the forward primer is for one
2 of exons 2 and 3 in a B locus of the HLA allele.

1 14. The primer of claim 11 wherein the forward primer is for exon
2 1 in a B locus of the HLA allele.

1 15. The primer of claim 11 further comprising one or more additional
2 primers that are complementary to one or more additional HLA alleles of interest.

1 16. A method for detecting the presence of an HLA allele
2 comprising:
3 amplifying a nucleic acid wherein the amplification reaction comprises
4 a primer set capable of amplifying one or more HLA alleles and a control primer pair
5 that will produce an HLA control amplicon of predetermined size from one or more
6 HLA alleles if the HLA alleles are present in the sample; and
7 detecting the presence of the HLA allele.

1 17. The method of claim 16 wherein the portion of the HLA allele
2 amplified by the control primer pair is common to all or substantially all HLA alleles.

1 18. The method of claim 16 wherein the portion of the HLA allele
2 amplified by the control primer pair comprises a portion of exon 4 of the HLA A
3 locus or HLA B locus.

1 19. The method of claim 16 wherein the HLA control amplicon is
2 about 500 to 1000 base pairs in length.

1 20. The method of claim 16 wherein the one or more HLA alleles
2 are common to a single HLA locus.

1 21. A method for amplifying a class I HLA allele comprising:
2 performing a nucleic acid amplification reaction on a sample having or
3 suspected of having a class I HLA allele wherein the nucleic acid amplification
4 reaction comprises the primer set of claim 11.

1 22. The method of claim 21 further comprising sequencing any
2 resulting HLA amplicons.

1 23. A method for determining the sequence of an HLA allele
2 comprising:
3 performing a sequencing reaction on an HLA allele using a forward
4 primer comprising a 3' portion and a 5' portion wherein the 3' portion is
5 complementary to an HLA allele of interest and the 5' portion is not complementary
6 to the HLA allele of interest wherein the primer allows complete resolution of an
7 exonic sequence; and
8 determining the sequence of one or more HLA alleles.

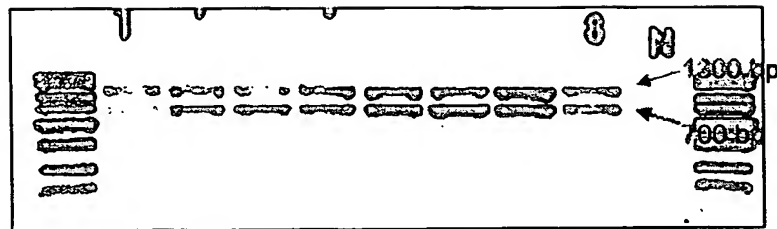
**PRIMERS, METHODS AND KITS FOR AMPLIFYING OR
DETECTING HUMAN LEUKOCYTE ANTIGEN ALLELES**

ABSTRACT OF THE INVENTION

5 The present invention describes primers, primer sets, methods and kits
for identifying and/or detecting HLA alleles. Using these primers and primer sets, all
HLA alleles at a single locus can be amplified. During HLA locus amplification,
control primer pairs may be used that produce control amplicons from the HLA allele
only if the particular HLA locus or allele is present in the sample. The present
invention also describes primers for sequencing HLA alleles following amplification.

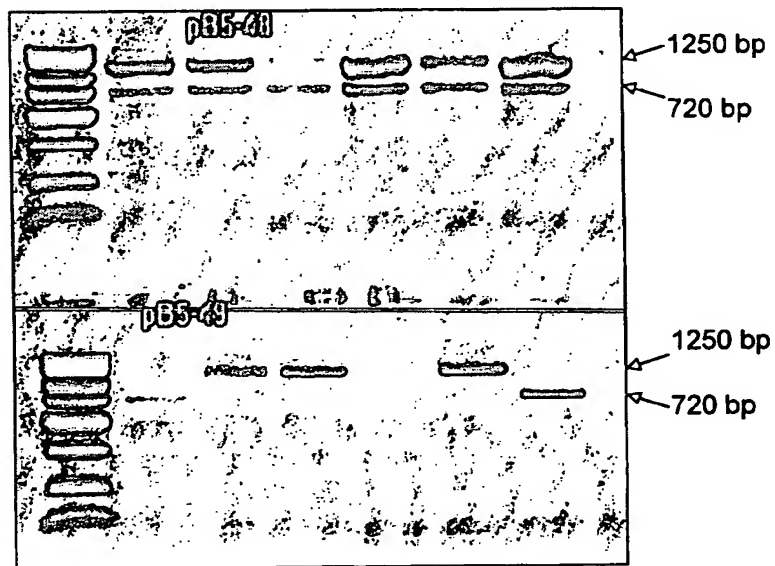
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FIGURE 1A



MW Markers

FIGURE 1B



MW Markers

2/2

FIGURE 2A

A Locus - exon 2 forward sequencing primer

¹⁶⁰ C A T C G C A G T G G ¹⁷⁰ G C T A C G T G G A C G A C A C G C A G ¹⁸⁰ T T C G T G C G G T T C G ¹⁹⁰ ²⁰⁰

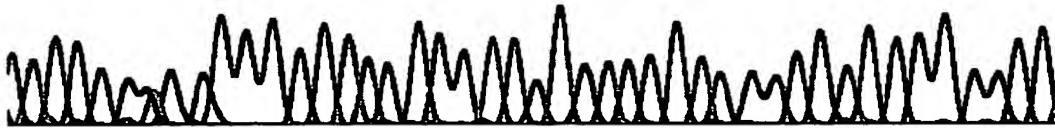


FIGURE 2B

A Locus - exon 2 reverse sequencing primer

⁴⁰ T C G C A G T G G G C T A C G T G G A C G A C A C G C A G T T C G T G C G G T T C G A ³⁵⁰ ³⁶⁰ ³⁷⁰ ³⁸⁰

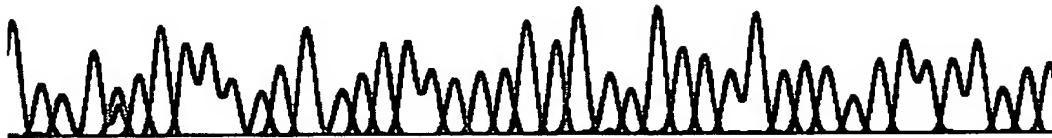


FIGURE 2C

B Locus - exon 2 forward sequencing primer

A C C G G A A C A C A C A G A T C T V C A A G R C C H A S R C A C A G A C T G A C C G A G ¹⁹⁰ ²⁰⁰ ²¹⁰ ²²⁰

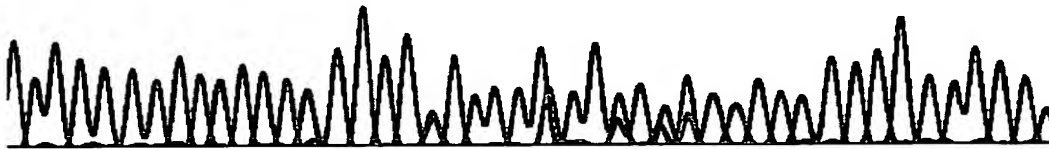


FIGURE 2D

B Locus - exon 2 reverse sequencing primer

C C G G A A C A C A C A G A T C T V C A A G R C C H A S R C A C A G A C T G A C C G A G ²⁹⁰ ³⁰⁰ ³¹⁰ ³²⁰



Application Data Sheet

Application Information

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Subject Matter::	Utility
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Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
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Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::

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